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PROTOZOOLOGY - ORIGINAL PAPER

First molecular survey of piroplasm species in cattle from Kyrgyzstan

Münir Aktaş[1] · İhsan Kısadere[2] · Sezayi Özübek[1] · Hüseyin Cihan[3] · Ruslan Salıkov[4] · Veli Yılgor Cirak[5]

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Abstract

Bovine piroplasmosis is a tick-borne disease caused by apicomplexan hemoparasites of the genera *Theileria* and *Babesia*. This study was carried out to assess the presence and frequency of piroplasm parasites in apparently healthy cattle in Kyrgyzstan. A total of 454 blood samples were collected from animals of various ages in eight villages located in the Chu valley and around the Lake Issyk Kul. The hypervariable V4 region of the 18S ribosomal RNA (rRNA) gene was amplified with a set of primers specific targeting members of the genera *Theileria* and *Babesia*. Amplified PCR products were hybridized onto a membrane to which generic and species-specific oligonucleotide probes were covalently linked. The results revealed the presence of three piroplasm species (*Theileria orientalis*, *Babesia major*, *Theileria annulata*). *Theileria orientalis* was the most prevalent species (32.8%; CI 28.5–37.3). *Babesia major* was the only species of *Babesia* found in any of the samples (1.3%; CI 0.5–2.8). The co-existence of *Theileria annulata* and *T. orientalis* was detected in nine animals (1.9%; CI 0.9–3.7). BLAST search revealed that the *Theileria* sequences shared 100% identity with the recently reported sequences for *T. buffeli* and *T. annulata*. The sequence of *B. major* was also 100% identical to an existing *B. major* sequence. This molecular survey provides important epidemiological data for control of bovine piroplasmosis caused by *T. orientalis*, *B. major*, and *T. annulata* in Kyrgyzstan.

Keywords Theileria · Babesia · PCR · Reverse line blot · Cattle · Kyrgyzstan

Introduction

Bovine piroplasmosis is a tick-borne disease caused by intra-erythrocytic pathogens of the genus *Babesia* and *Theileria* (phylum Apicomplexa). These protozoan parasites cause significant economic losses in cattle in tropical and subtropical countries (Bock et al. 2004). Among the main *Babesia* species infecting cattle, *Babesia bigemina*, *B. divergens*, and *B. bovis* cause severe clinical cases and fatal diseases in untreated animals whereas *B. major* and *B. occultans* are less pathogenic and often cause subclinical infection (Aktas and Ozubek 2015). *Theileria annulata* is considered as the causative agent of tropical theileriosis, whereas *T. orientalis* complex causes mild or subclinical infections in cattle (Aktas et al. 2006). However, recent studies revealed that some genotypes of *T. orientalis* cause severe clinical infections in Australia and New Zealand (Gebrekidan et al. 2017a).

Traditionally, identification of hemoprotozoan parasites is based on thin or thick blood smears stained with Giemsa. However, this technique often lacks specificity to distinguish between piroplasmid species that co-infect the same host. Therefore, detection of these parasites requires the use of a simultaneous identification method such as reverse line blot (RLB) hybridization (Altay et al. 2012; Iqbal et al. 2013; Aydin et al. 2013).

Kyrgyzstan is a landlocked country, located in Central Asia, and exhibits alpine and subalpine habitats (Frenken 2013). *Ixodes persulcatus*, *Haemaphysalis punctata*, *Haemaphysalis erinacei*, *Dermacentor marginatus*, *Rhipicephalus pumilio*, *Rhipicephalus sanguineus*, *Rhipicephalus turanicus*, and *Hyalomma anatolicum* have been reported in Bishkek,

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✉ Münir Aktaş
maktas@firat.edu.tr; vetmaktas@gmail.com

[1] Department of Parasitology, Faculty of Veterinary Medicine, Firat University, 23119 Elazig, Turkey

[2] Department of Physiology, Faculty of Veterinary Medicine, Balikesir University, Balikesir, Turkey

[3] Department of Internal Medicine, Faculty of Veterinary Medicine, Bursa Uludag University, 16059 Bursa, Turkey

[4] Department of Pharmacology and Toxicology, Faculty of Veterinary Medicine, Kyrgyz-Turkish Manas University, 720044 Bishkek, Kyrgyzstan

[5] Department of Parasitology, Faculty of Veterinary Medicine, Bursa Uludag University, 16059 Bursa, Turkey

Kyrgyzstan (Fedorova 2005). However, there has been no report on the distribution of tick-borne bovine piroplasms in the country. In this study, we performed a PCR-based RLB survey on apparently healthy cattle to assess the occurrence and frequency of piroplasm parasites in Kyrgyzstan.

Materials and methods

Study area and sample collection

This work was performed on cattle in the eight villages (Tokmok, Sokuluk, Karashar, Kyzyl-Töbö, Kopuro Bazar, Tamga, Kayyngdy, Moldovanovka) located in the Chu valley and around the Lake Issyk Kul of Kyrgyzstan (Fig. 1). This area has a continental climate and it can get quite hot at low altitudes, but cool in the mountains (Frenken 2013). A total of 454 blood samples from cattle were taken in tubes containing ethylendiamine tetraacetic acid (EDTA) with the cooperation of Kyrgyz-Turkish Manas University, Veterinary Faculty academic staffs. The sampling was carried out from December 2012 to June 2013. The sampled cattle were divided into categories based on age (< 1 year or > 1 year) and sex (male and female). They were apparently healthy, but particular clinical examination was not performed. As the animals were not examined for ticks, no data were available.

DNA extraction, PCR amplification, and reverse line blotting

Total DNA extraction from blood samples was performed using a genomic DNA mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. For screening analysis, piroplasm DNA was detected by PCR based on the V4 hypervariable region of the 18S rRNA gene using the primer pair RLB-F2 and RLF-R2 as described (Georges et al. 2001). The PCR amplification was performed as reported by Ozubek and Aktas (2017). Positive control DNAs from *T. orientalis*, *T. annulata*, and *B. bigemina* (GenBank accession nos. EU622821, HQ646253, and KP745623, respectively) were used in the assay.

Reverse line blotting was performed as reported by Aydin et al. (2013). Briefly, to 20 μL of the amplification products, 2X SSPE/0.1% SDS was added to a final volume of 150 μL, held in the Thermal Cycler at 99 °C for 10 min, and denatured for the assay. The amplicons were then hybridized with the *Theileria*/*Babesia* catch-all, *Theileria* spp., *Babesia* spp., and species-specific oligonucleotide probes (*B. bovis*, *B. bigemina*, *B. divergens*, *B. major*, *B. occultans*, *T. annulata*, and *T. buffeli/orientalis*) linked to an RLB membrane. The probes were synthesized by The Midland Certified Reagent Co., Inc., (USA) and used with a concentration range of 200–400 pmol/150 μL. One hundred fifty microliter of each sample was loaded onto the membrane, using a

Fig. 1 Map of Kyrgyzstan, showing the sampling villages

Table 1 Prevalence of piroplasm infections in Kyrgyzstan

Villages	*n* (%)	Single infection (%)			Mixed infection (%)
		T. orientalis	*B. major*	*T. annulata*	*T. orientalis* + *T. annulata*
Maldovanovka	60/65 (92.3%)[a]	60 (92.3%)[a]	–	–	–
Tokmok	13/20 (65%)[b]	13 (65%)[b]	–	–	–
Sokuluk	29/57 (%50.8)[b]	22 (38.6%)[b]	–	–	7 (12.3%)
Karashar	3/42 (7.1%)[b]	3 (7.1%)[b]	–	–	–
Kyzyl-Töbö	6/76 (7.9%)[b]	3 (3.9%)[b]	3 (3.9%)	–	–
Kopuro Bazar	21/98 (21.4%)[b]	19 (19.4%)[b]	–	–	2 (2%)
Tamga	5/54 (9.2%)[b]	2 (3.7%)[b]	3 (5.5%)	–	–
Kayyngdy	27/42 (64.3%)[b]	27 (64.3%)[b]	–	–	–
Total	164/454 (36.1%)	149 (32.8%)	6 (1.3%)	–	9 (1.9%)

Different superscript letters (a, b) denote significantly different ($p < 0.05$) prevalence of piroplasms *T. orientalis*

Miniblotter MN45 (Immunetics, MA, USA). Hybridized PCR products were detected by chemiluminescence reactions using ECL reagents (Amersham, UK) after the labeling of biotin with streptavidin horseradish peroxidase. Reactions were visualized using the c-Digit imaging system (LI-COR Biosciences, Lincoln, NE). Black spots occurring in rows where PCR products and probes were crossed were evaluated as positive to the related species.

The membrane was washed twice in 1% SDS for 30 min at 85 °C to strip PCR products from the membrane. Finally, the membrane was rinsed in 20 mM EDTA for 15 min at room temperature and stored in 20 mM EDTA at 4 °C for reuse.

Sequencing

To verify RLB findings, PCR amplicons comprising *B. major*, *T. annulata*, and *T. orientalis* were selected for sequencing. DNA amplicons were purified with a PCR purification kit (Qiagen, Hilden, Germany), and the 18S rRNA gene fragments were sequenced with forward primers. Each construct was sequenced at least three times. The nucleotide sequences of these amplicons were determined using an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, USA). BLASTn analysis was performed to identify identical and/or similar sequences in the database.

Statistical analysis

The Pearson chi square (χ^2) test was performed using the SPSS 15.00 software to compare the data obtained from cattle for correlated proportions. Association of piroplasms with sex and age was assessed. p values ≤ 0.05 were evaluated to be statistically significant.

Results

Occurrence and frequency of piroplasm infections in cattle

The amplification products were hybridized onto the membrane. Occurrence of single and combined piroplasm infections are presented in Table 1. In the PCR assay, 164 out of 454 (36.1%) examined blood samples were found to be infected with *Theileria* or *Babesia* species. The highest number of positive samples was obtained from the village of Maldovanovka (92.3%), followed by Tokmok (65%), Kayyngdy (64.3%), and Sokuluk (50.8%). The lowest prevalence of piroplasms was in Karashar (7.1%), and the difference between Maldovanovka and other villages was significant ($p < 0.05$).

PCR-based RLB revealed the presence of three piroplasm species (*T. orientalis*, *B. major*, *T. annulata*). *Theileria orientalis* was the most prevalent species (32.8%). It was detected in all villages. *B. major* was the only species of *Babesia* found in any of the samples (1.3%). The co-existence of *T. annulata* and *T. orientalis* was detected in nine animals (1.9%).

The frequency of piroplasm infections in cattle with respect to age and gender is shown in Table 2. Statistical differences were neither found between young and adult ($p = 0.902$) nor between male and female cattle ($p = 0.457$).

DNA sequencing

To confirm the RLB results, the partial sequences of *T. annulata*, *T. orientalis*, and *B. major* were determined and submitted to the GenBank (MK415835, MK415836, and MK415837, respectively). BLAST search showed that the *Theileria* sequences shared 100% identity with the recently reported sequences for *T. orientalis* (MH327771) and

Table 2 Association of the presence (RLB-positive, %, 95% CI, and *p* value) of piroplasms in cattle with age and gender

	n	Age		Gender	
		< 1 year	> 1 year	Male	Female
No. sample	454	112	342	80	374
Positive	164	41	123	26	138
%	36.1	36.6	36.4	32.5	36.9
95% CI	31.7–40.7	27.7–46.2	30.9–41.3	22.4–43.9	32.0–42.0
p value		> 0.05		> 0.05	

T. annulata (MK183002). The sequence of *B. major* showed 100% identity to an existing *B. major* sequence (GU194290).

Discussion

Livestock is a significant part of the Kyrgyzstan's economy. The majority of its population derive their livelihood from livestock and agriculture (Zhumanova and Maharjan 2012). Tick-borne infections have great economic impact on livestock worldwide, resulting in abortion, weight loss, decreased meat and milk production, and death (Aktas and Ozubek 2015). Bovine piroplasmosis has been reported in many Central Asian countries (Gralén 2009). However, there is no available molecular data on the occurrence of *Theileria* and *Babesia* parasites in cattle in Kyrgyzstan. The present study provides the first molecular survey of the genera *Theileria* and *Babesia* species, and a relatively high prevalence of 36.1% was detected in sampled cattle.

In this study, *B. major* was the only *Babesia* species detected in apparently healthy cattle, but at a lower prevalence of 1.3%. The low prevalence of *B. major* is in agreement with previous studies from other European countries, including Turkey (Altay et al. 2008) and Hungary (Hornok et al. 2014). *B. major* was identified from cattle in the Xinjiang region of northwestern China, which is bounded by Kyrgyzstan, and the three piroplasm species *T. orientalis*, *T. annulata*, and *B. major* were identified (Yin et al. 1996). Furthermore, piroplasm infection was found to be equally distributed in young and adult as well as in male and female cattle (Yin et al. 1996). In a recent tick survey, *B. major* was detected in *Haemaphysalis punctata* collected from sheep in the same region of China (Song et al. 2018). These findings extend the area of occurrence for *B. major*, and indicate that it is common in the central Asia and Far East. No other pathogenic *Babesia* species infecting cattle (*B. bovis*, *B. bigemina*, *B. divergens*) were detected in the current survey. This finding contrasts with a recent report in which *B. bigemina*, *B. bovis*, and *B divergens* have been reported to be common in neighboring countries of Kyrgyzstan (Hassan et al. 2018).

Our study confirms the presence of bovine theileriosis in Kyrgyzstan. Surprisingly, in our survey, single infection with *T. annulata* was not detected, but it was observed always as mixed infection with *T. orientalis* in a low prevalence of 1.9%. The low prevalence of *T. annulata* disagrees with previous studies with neighboring of Kyrgyzstan (Rasulov et al. 2008). The highest frequence of single infection was recorded for *T. orientalis*, with a RLB-estimated prevalence of 32.8%. The high prevalence of oriental theileriosis as compared with other pathogens in our survey can be explained by the fact that hosts are usually infected with this mild pathogenic parasite at an early age and they remain life-long carriers (Sugimoto and Fujisaki 2002). The prevalence of oriental theileriosis obtained in this study was higher than that of reports from the Pakistan with 24.5% (Gebrekidan et al. 2017b) and Vietnam with 13.8% (Khukhuu et al. 2011), but lower than reported in the Asia-Pacific region such as Mongolia 41.7% (Altangerel et al. 2011), Japan 64.8% (Ota et al. 2009), Sri Lanka 53.5% (Sivakumar et al. 2013), and Myanmar 36.2% (Bawm et al. 2014). The observed variations in prevalence could be attributed to the origin of cattle imported from countries endemic for oriental theileriosis (Gebrekidan et al. 2017a, b). In addition, the discrepancies in the prevalence of the disease among different countries may also be attributed to variation of management practices, tick abundance in the sampling region and intensity in the sampling region, molecular test and markers employed, susceptibility of animal breeds, the presence of reservoirs, as well as ecological and climatic conditions.

In conclusion, this is the first molecular epidemiological study of bovine piroplasmosis in Kyrgyzstan, and has identified three piroplasm species (*T. orientalis*, *T. annulata*, and *B. major*).

Compliance with ethical standards

Commission of ethics All procedures were approved by the Ethical and Animal Welfare Committee from the Kyrgyz-Turkish Manas University, under protocol number 2012-03/1.

Conflict of interest The authors declare that they have no conflict of interest.

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